

**Estradiol Protects Against Ethanol-Induced Bone Loss by Inhibiting Up Regulation of  
RANKL in Osteoblasts**

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List of abbreviations: E<sub>2</sub>, 17 $\beta$ -estradiol; RANKL, receptor activator of NF-kappaB ligand; OPG, osteoprotegerin; pQCT, peripheral quantitative computer tomography; BMD, bone mineral density; BMC, bone mineral content; EtOH, ethanol; ADH, alcohol dehydrogenase; TEN, total enteral nutrition; TRAPase, tartrate resistant acid phosphatase; ADH1, Class 1 alcohol dehydrogenase; 4-MP, 4-methylpyrazol; ERK, extracellular signal-regulated kinase; STAT3, signal transducers and activators of transcription 3; CYP2E1, cytochrome P450 2E1.

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**ABSTRACT**

To investigate the effects of sex hormones on ethanol (EtOH)-induced bone loss, female Sprague-Dawley rats were fed control or EtOH-containing diets (12 g/kg/d) by intragastric infusion. After 3 weeks, rats receiving EtOH had significant decreases in tibial trabecular and total bone mineral density; induction of RANKL mRNA expression and enhanced bone resorption, all of which were prevented by treatment with 17- $\beta$  estradiol (E<sub>2</sub>). Addition of progesterone did not enhance the beneficial effect of E<sub>2</sub> alone. Consistent with our *in vivo* findings, EtOH stimulated RANKL mRNA expression in cultured primary osteoblasts and this was blocked by 4-methylpyrazole. Acetaldehyde also induced RANKL expression. Class 1 alcohol dehydrogenase was found to be expressed and EtOH-inducible in cultured osteoblasts; whereas CYP2E1 was undetectable. We found that EtOH induced phosphorylation of extracellular signal-regulated kinase (ERK) and signal transducers and activators of transcription 3 (STAT3). E<sub>2</sub> and the MEK inhibitor PD98059 blocked ERK and STAT3 phosphorylation and blocked RANKL induction. Moreover, E<sub>2</sub> completely blocked EtOH-induced osteoclastogenesis in a primary osteoblast and osteoclast precursor co-culture system. The E<sub>2</sub> effects were estrogen receptor-mediated. Therefore, E<sub>2</sub> prevents EtOH-induced bone loss by opposing the induction of RANKL mRNA in osteoblasts and ethanol-induced osteoclastogenesis, through opposing effects on sustained ERK signaling.

## INTRODUCTION

Chronic ethanol (EtOH) abuse is well known to result in osteoporosis and increased fracture risk in men and women (Chakkalakal, 2005). Moreover, an inverse correlation between EtOH intake and bone mineral density has been reported in both pre- and post-menopausal women (Turner and Sibonga, 2001). The molecular mechanisms whereby EtOH consumption results in osteotoxicity are not well understood. Appropriate bone remodeling depends on a balance between the actions of osteoclasts which can remove old bone and osteoblasts which are responsible for forming new bone (Manolagas, 2000). In the bone marrow, mesenchymal stem cells are capable of differentiating into multiple mature cell types including osteoblasts (Zuk, 2001). As multifunctional cells, osteoblasts also control the differentiation of osteoclasts. Hematopoietic stem cells develop into osteoclasts in response to the presence of the receptor activator of NF-kappaB ligand (RANKL) expressed on the surface of osteoblasts which binds to the protein RANK expressed on the surface of osteoclast precursors. This process is modulated by osteoprotegerin, an endogenous inhibitor of RANK-RANKL signaling (Quinn et al., 2000). After osteoclasts are differentiated, their activity is regulated by a variety of hormones, growth factors, and cytokines (Teitelbaum, 2000). Alcohol abuse may promote bone loss through both inhibition of osteoblastogenesis (Friday and Howard, 1991) and induction of osteoclast differentiation and activation (Cheung et al., 1995). Dai et al. suggested that EtOH increases RANKL mRNA expression in bone marrow cells resulting in stimulation of osteoclastogenesis and bone resorption mediated via induction of IL-6 (Dai et al., 2000).

Disruption of sex steroid homeostasis, including a reduction in serum estradiol, is also a reported feature of young female alcoholics (Dorgan et al., 1994). Sex steroids have been reported to protect against bone loss and it is possible that impaired sex steroid signaling contributes to

completely blocked by *in vivo* supplementation with either E<sub>2</sub> alone or E<sub>2</sub> + P ( $P < 0.05$ ) (Fig. 2A). Osteoprotegrin is an endogenous inhibitor of RANK-RANKL signaling which modulates the bone resorption process. Osteoprotegrin mRNA levels were not altered by EtOH, E<sub>2</sub> or E<sub>2</sub> + P (Fig. 2B). Osteocalcin mRNA levels were significantly decreased in EtOH-treated groups, but E<sub>2</sub> or E<sub>2</sub> + P did not reverse these effects. (Fig. 2C).

### **EtOH Induces RANKL mRNA Expression in Differentiated Primary Osteoblasts *in vitro*.**

We performed *in vitro* studies to establish a cellular mechanism for our *in vivo* findings. Bone marrow cells were taken from the femur of untreated cycling 250-300 g Sprague-Dawley female rats. Differentiated osteoblasts were generated after 20 days in culture according to previous methods (DiGregorio et al., 2001). Primary osteoblasts treated with 25 or 50 mM EtOH for different times had a dose-responsive increase in RANKL mRNA expression as measured by real-time RT-PCR ( $P < 0.05$ ) (Fig. 3A). RANKL mRNA expression was highest at 12-24 h following the beginning of treatment and declined thereafter ( $P < 0.05$ ). RT-PCR analysis revealed that mRNA encoding ADH I, the main EtOH metabolizing enzyme, was detected in osteoblasts and quantitation by real time RT-PCR confirmed that this mRNA was inducible by EtOH ( $P < 0.05$ ) (Fig. 3B,3D). The decline in RANKL mRNA expression followed induction of ADH I and the disappearance of EtOH in the culture medium as a result of metabolism (Fig. 3C). An EtOH concentration of 50 mM *in vitro* is equal to 230 mg/dl, and was similar to that attained in our study *in vivo*. RT-PCR analysis demonstrated that the other important enzyme involved in EtOH oxidation, CYP2E1, was not detectible in either EtOH-treated or EtOH-non-treated differentiated primary osteoblasts even after 30 cycles of amplification (Fig. 3D).

To test whether EtOH had direct effects on induction of RANKL expression in osteoblasts or if it needed to be metabolized to exert its action, we pre-treated the cells with the ADH I inhibitor 4-

induced loss of bone density are mainly due to its inhibitory effects on osteoclastogenesis or osteoclast activity because BMD was restored by E<sub>2</sub> treatment even though the bone formation marker osteocalcin remained suppressed. The effects of E<sub>2</sub> and EtOH on osteocalcin were complex. Although no further decrease on osteocalcin mRNA were observed, plasma concentrations were lower following combined treatment. This may reflect post-transcriptional effects or effects on osteocalcin secretion. The increase of serum P concentrations with the combination of E<sub>2</sub> + P in EtOH-treated rats may be due to impaired P metabolism or clearance (Hidestrand et al., 2005; Sugano et al., 1995).

Experiments using an *in vitro* cell culture model of differentiated primary osteoblasts and osteoblast/osteoclast precursor co-cultures further characterized the cellular and molecular mechanisms underlying the opposing effects of EtOH and E<sub>2</sub> on bone. We have demonstrated for the first time that E<sub>2</sub> acting through the estrogen receptor can inhibit the induction of the osteoclast differentiation factor RANKL and directly interfere with EtOH-induced osteoclastogenesis. However, the signal transduction mechanisms underlying these processes especially in bone cells are poorly understood, and need to further explored.

Until now, it has been suggested that only liver and gastric mucosa have sufficiently high expression of isoforms of alcohol dehydrogenase to be involved in *in vivo* EtOH clearance (Lieber, 1993) and that local metabolism of EtOH by other tissues including bone marrow is primarily associated with a cytochrome P450 enzyme, CYP2E1 (Ronis et al., 1996; Bernauer et al., 2000). The data in this study demonstrate that ADH I was significantly expressed in differentiated osteoblasts and that these cells are able to metabolize EtOH locally to acetaldehyde in a manner analogous the liver. Indeed, EtOH disappearance in the osteoblast cultures coincided with induction of ADH mRNA expression by EtOH and reversal of the EtOH induction of

RANKL mRNA expression. In contrast, no expression of CYP2E1 mRNA was detected in differentiated osteoblasts. The ADH class I inhibitor 4-MP was able to completely abolish EtOH-induced RANKL expression in osteoblasts, while the primary EtOH metabolite acetaldehyde was also able to stimulate RANKL mRNA expression. These data suggest that EtOH metabolism to acetaldehyde in osteoblasts is required to produce increases in RANKL expression.

Our present study also showed that EtOH-induced RANKL mRNA expression is downstream of the phosphorylation of ERK and STAT3. These data further suggest that EtOH has its effects on bone remodeling as a result of sustained activation of protein kinases. It is becoming clear that MAP kinase molecules including ERK are involved in the signal transduction of a variety of cellular responses including proliferation, differentiation, survival and execution of inflammatory responses (Aroor and Shukla, 2004; Cross et al., 2000). In agreement with our observation of sustained increases in ERK phosphorylation in osteoblasts, EtOH has been reported to cause gradual and sustained activation of ERK in both primary rat hepatocytes and liver *in vivo* and this prolonged activation of ERK was shown to play a prominent role in cell cycle arrest (Cross et al., 2000). It is well known that E<sub>2</sub> can acutely activate ERK in osteocytes *in vitro* (Chen et al., 2005). However, this is a rapid and transient effect occurring over a time scale of minutes followed by a return to base values after 2 hours. We present data in the current study that chronic *in vitro* E<sub>2</sub> treatment attenuates EtOH-induced ERK phosphorylation over a period of 12-48 hours. This suggests that ERK may play an important role in modulation of both EtOH and E<sub>2</sub> signaling in osteoblasts. Sustained ERK activation in response to EtOH exposure *in vivo* activates nuclear transcription factors and alters gene expression within the liver, leading to the development of steatosis and inflammation in the early stages of EtOH-induced liver injury (Nagy, 2004). We speculate that some actions of EtOH in bone marrow may be analogous to the